BEAN YELLOW MOSAIC VIRUS COAT PROTEIN AND *GUSA* GENE EXPRESSION IN TRANSGENIC *GLADIOLUS* PLANTS

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Abstract

Transgenic plants of *Gladiolus* were created following particle gun bombardment with either the bean yellow mosaic virus coat protein or *gusA* gene each under control of the CaMV 35S promoter. Monoclonal antibodies were used in ELISA assays to detect expression of the bean yellow mosaic virus coat protein in leaves of the transgenic plants. The bean yellow mosaic virus coat protein was expressed in 42% of the transgenic plants grown *in vitro* for 1 1/2 years, and levels of coat protein expression remained similar for each transformed plant. Coat protein was expressed in plants grown in the greenhouse after two seasons of dormancy, and expression levels exceeded that of plants grown *in vitro*. Plants expressing *gusA* were developed to determine levels of reporter gene expression in various plant tissues and in independent transformants as controlled by the CaMV 35S promoter. The strongest GUS expression generally occurred in root meristems as determined by histochemical staining. GUS expression was variable between leaves of a plant. Leaves of plants developed by particle gun bombardment of suspension cells showed a more uniform distribution of GUS expression throughout the length of the leaf compared to plants resulting from transformation of cormel slices that typically exhibited a distinct striped pattern of GUS expression.

1. Introduction

Gladiolus ranked fifth in 1993 for the number of cut flower stems (79,663 million) shipped worldwide (US Department of Agriculture, 1994). A major problem for *Gladiolus* growers is viral infection resulting in decreased plant vigor and visual viral symptoms making the flowers unmarketable. Bean yellow mosaic virus (BYMV) is ubiquitous in *Gladiolus* (Stein, 1995). Recent advances in genetic engineering for virus resistance has made *Gladiolus* an ideal target for improvement by developing transgenic plants. Transgenic *Gladiolus* plants containing the BYMV gene under the CaMV 35S promoter have been developed. This study characterized the levels of BYMV coat protein as affected by dormancy and time *in vitro*. Coat protein levels differed between transgenic plants. Tissue-specific and levels of gene expression regulated by the CaMV 35S promoter was determined using the GUS reporter gene.

2. Materials, methods and experiments

2.1. Tissue culture

Regenerable callus was initiated from either cormel slices or *in vitro*-grown plantlets of *Gladiolus* (Kamo, *et al.*, 1990; Logan and Zettler, 1985). Callus was grown on Murashige

and Skoog's (MS) basal salts medium (Murashige and Skoog, 1962) supplemented with 2% sucrose, 0.2% Gelrite and the following (mg/liter) glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; and either dicamba, 2.0; α-napthaleneacetic acid, 10.0, or 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5. Suspension cells were initiated from the callus and cultured in the same medium as for callus culture, except that Gelrite was omitted. Both callus and suspension cells were grown in the dark at 25 °C.

2.2. Plasmid constructs

Plasmid DNA was isolated using alkaline lysis followed by cesium chloride gradient centrifugation (Maniatis, *et al.*, 1982). Plasmid p35SAc contains the PAT gene under the CaMV 35S promoter. Both the BYMV coat protein and *gusA* genes were cloned under the CaMV 35S promoter.

2.3. Particle gun bombardment

Suspension cells were bombarded by Sanford Scientific, Inc. using the BioRad PDS-1000-He instrument (Sanford, *et al.*, 1991; 1993). M10 tungsten particles were coated with plasmid DNA, and the cells were bombarded at 8.3 MPa (1200 psi) one time per plate. The gap distance was 1 cm and target distance was 12 cm.

2.4. Selection

Selection is described in detail (Kamo, *et al.*, 1995). Briefly, suspension cells were exposed to a selective agent, either phosphinothricin (PPT) or bialaphos, one week after particle gun bombardment. Cells were cultured on solidified MS basal salts medium supplemented with 0.5 mg/liter 2,4-D and either PPT or bialaphos at either 1 or 6 mg/liter. Cells were grown in the dark for 3-6 months until the majority of cells had died. Remaining cells were cultured on regeneration medium consisting of MS basal salts, 2 mg/liter kinetin and bialaphos at either 0, 0.5, or 3 mg/liter bialaphos. Regeneration cultures were grown under a 16-h light photoperiod. Shoots were transferred to MS basal salts medium lacking hormones.

2.5. BYMV coat protein analysis

Coat protein was detected using monoclonal antibodies to BYMV in an ELISA assay (Jordan and Hammond, 1991). Plant leaves were ground in liquid nitrogen in coating buffer supplemented with 2% PVP (PVP M_r 40,000) using 10 ml buffer/g fresh weight leaves. The supernatant was filtered with Miracloth, and centrifuged at 10,000 x g for 10 min at 4 °C. and then diluted either 10 or 50-fold and used for coating of the ELISA plate wells. After a 16 h incubation at 4 °C of antigen in the ELISA plate, the wells were washed three times with TBS (20 mM Tris-HCl pH 7.5, 0.15 NaCl) containing 0.05% Tween 20. Wells were then filled with TBS supplemented with 1% dry milk and 0.5% BSA and incubated for 1 h at 25 °C followed by a 16 h incubation at 4 °C with hybridoma culture supernatant. Next, wells were washed three times with TBS and then filled with a goat anti-mouse IgG, IgA, IgM alkaline phosphatase conjugate for 3 h at 25 °C. Plates were then washed three times with TBS and then substrate buffer (1 mg/ml p-nitrophenyl phosphate in 0.1 M diethanolamine, 1 mM Mg_2Cl pH 9.8) added. Absorbance at A_{405} was read after 3 h. The two monoclonal antibodies PTY 1 and PTY 43 were used in duplicate in the same assay against each antigen.

2.6. GUS analysis

GUS activity was determined by histochemical and fluorimetric determination of plant tissues according to the method of Jefferson, *et al.* (1987).

3 Results

3.1. BYMV coat protein expression

Transgenic *Gladiolus* plants were developed by particle gun bombardment of suspension cells with the BYMV coat protein gene under the CaMV 35S promoter. Stable integration of the BYMV coat protein gene in *Gladiolus* plants was confirmed by Southern hybridization (data not shown). Transgenic plants were analyzed for BYMV coat protein expression by ELISA assay 9 months after particle gun bombardment. BYMV coat protein was expressed at various levels in leaves of transgenic *Gladiolus* plants grown *in vitro* (Figure 1). All four transgenic plants continued to express BYMV coat protein throughout two years of *in vitro* growth. Many of the transgenics (58%) did not express the BYMV coat protein in their leaves (Figure 2). *Gladiolus* plants transformed with the BYMV coat protein gene generally grew poorly *in vitro* as compared to non-transformed plants which grew rapidly on MS basal salts medium lacking hormones.

Cormels collected from *in vitro*-grown plants were used to start plants in the greenhouse. Transgenic plants grown in the greenhouse were phenotypically normal, however, the transgenic plants did not grow as vigorously as non-transformed plants. After each season's growth in the greenhouse, the corms were collected and placed in dormancy. The BYMV coat protein was expressed in *Gladiolus* plants after the first and second year of dormancy (Figure 3). In several plants the level of BYMV coat protein expression of greenhouse-grown plants exceeded the level from *in vitro*-grown plants.

3.2. GUS expression

Transgenic *Gladiolus* plants containing the *gusA* gene under the CaMV 35S promoter were developed by particle gun bombardment of suspension cells. Transformants were initially selected based upon GUS expression in a histochemical assay of callus, plant leaves and roots. One year after particle gun bombardment of the suspension cells, transgenic plants grown *in vitro* were assayed for GUS expression. Histological staining showed that leaves from 71% and roots from 86% of the plants expressed GUS. The fluorimetric assay determined that leaves from 80% of the transgenic plants expressed GUS. GUS was expressed most strongly in the root meristems and sometimes throughout the length of the root. Several leaves from a single plant grown *in vitro* showed a variable pattern of GUS expression. Some leaves showed strong GUS expression throughout the length of the leaf while other leaves from the same plant showed either no or very little expression exhibited as a streaked pattern. Previously transgenic *Gladiolus* plants were recovered following particle gun bombardment of cormel slices. Leaves taken from these plants showed a distinct striped pattern of GUS expression. This striped pattern was never seen in leaves derived from bombarded suspension cells.

4. Discussion

These transgenic *Gladiolus* plants are the first report of a large number of floral bulb plants that have been developed by genetic engineering. The BYMV coat protein gene was chosen for transforming *Gladiolus* because BYMV is ubiquitous in *Gladiolus* and the same

gene conferred virus resistance when used to develop transgenic tobacco plants (Hammond and Kamo, 1993). There was also no effect of the BYMV coat protein gene on growth of the transgenic tobacco plants as there was in *Gladiolus* plants. This demonstrates that unexpected differences can occur between plant species when using the same transgene. It appears that if genetic engineering of *Gladiolus* with BYMV coat protein is to be successful, the precise gene sequence that is responsible for conferring virus resistance should be used for transformation rather than full length coat protein. Possibly the transgenic *Gladiolus* plants were unable to express higher levels of BYMV coat protein than detected because higher levels of coat protein would be too detrimental to growth of the plant. There were times when the transgenic plants barely survived growth *in vitro*.

It was fortunate for future research involving genetic engineering of floral bulb crops that the BYMV coat protein was expressed in greenhouse-grown plants after the first and second season of dormancy. Flowering of *Gladiolus* occurs after 2 to 3 seasons of growth. A few flowers developed after the second season of growth, however, we were unable to get seed set so it is not known whether stable inheritance of the BYMV coat protein gene occurs.

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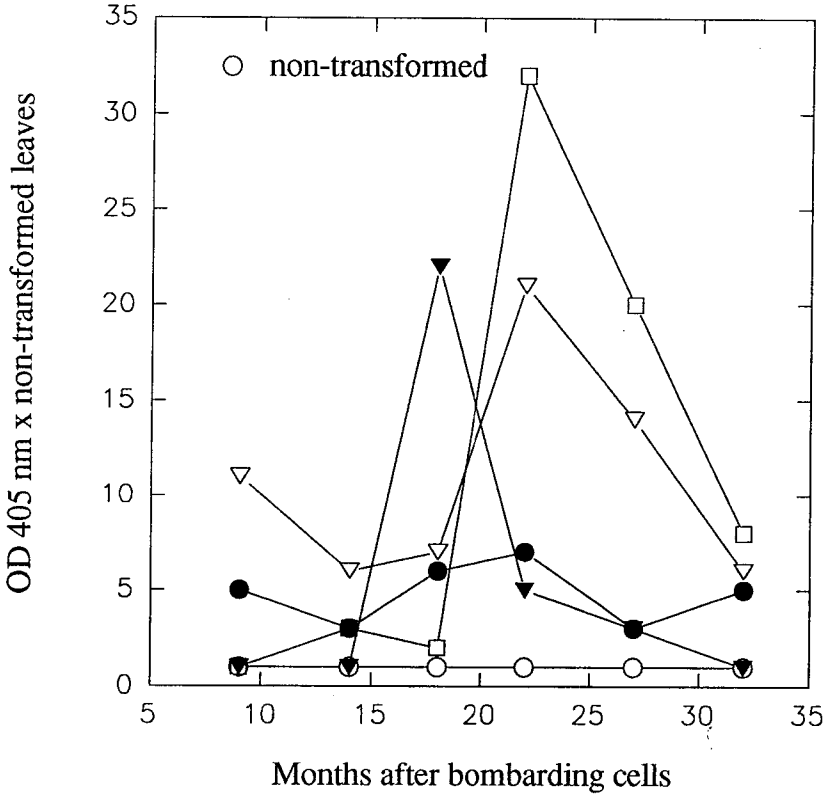

Figure 1. Levels of BYMV coat protein expression in four transgenic Gladiolus plants.

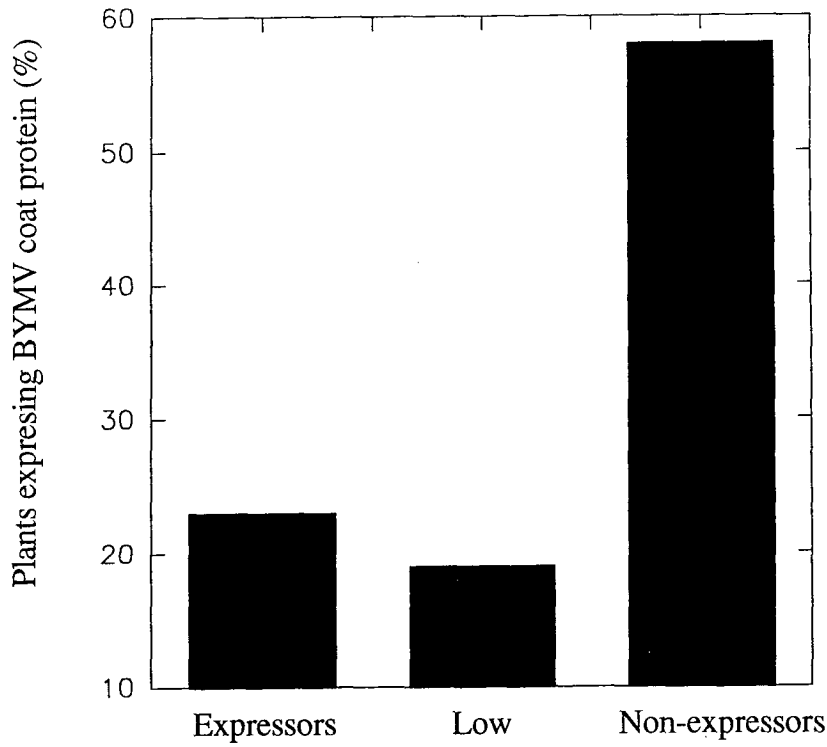

Figure 2. Number (%) trangenic Gladiolus plants expressing the BYMV coat protein in their leaves. There were 31 total plants. The "Low" level expressors were 2 - 3 times and "Expressors" were above 3 times the levels of non-transformed plants.

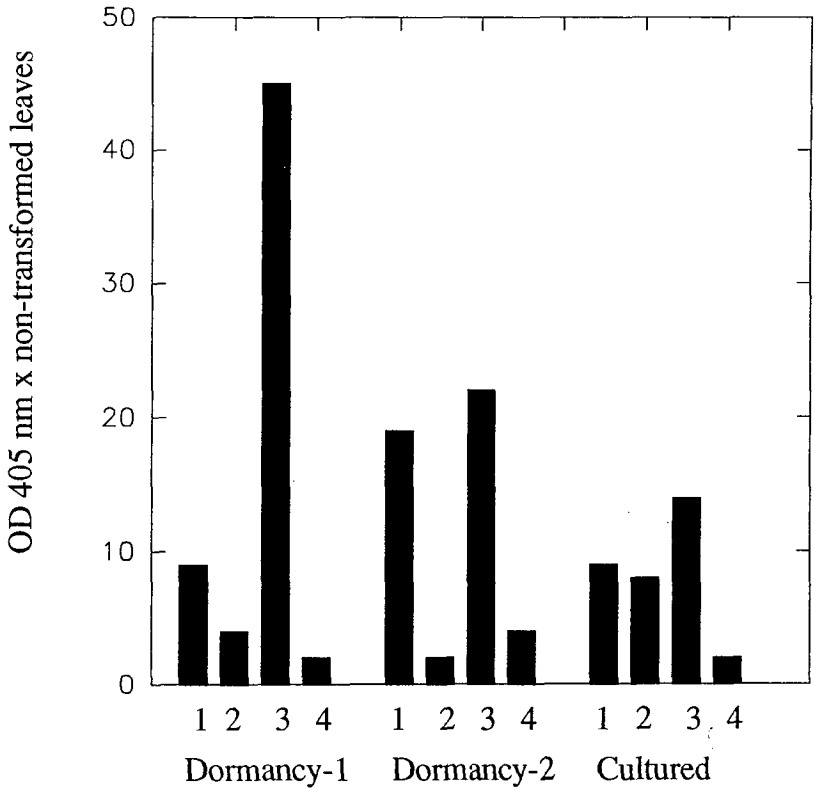

Figure 3. Levels of BYMV coat protein expression in four transgenic Gladiolus plants that were each grown in the greenhouse after the first and second years of dormancy and grown continuously in culture.